POTENTIAL FOR RAPID *IN VITRO* ASSAYS TO MEASURE FOODBORNE *SALMONELLA* VIRULENCE IN FOODS – A REVIEW

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Accepted for Publication October 1, 2004

ABSTRACT

Assessing the public health risk to consumers of foods is a priority in terms of food safety because of frequency of foodborne illness outbreaks caused by pathogenic bacteria such as Salmonella*. Innovative and rapid methods continue to be devised to detect, isolate and measure the potential for foodborne disease caused by foodborne* Salmonella *spp. in the U.S.A. To develop rapid* in vitro *assays for assessment of virulence, it is important to understand the physiological and genetic principles that enable* Salmonella *to become invasive and infective within a host. Combining this knowledge with rapid detection technologies will lead to more extensive and real time appraisal of* Salmonella *physiological and pathogenic status in food matrices.*

SALMONELLA-FOODBORNE DISEASE

Salmonella are especially important because of their prevalence in a wide variety of foods from animal origin and even produce. *Salmonella enterica* subspecies *enterica* serovar Typhimurium (identified as *Salmonella* Typhimurium) is generally considered to be one of the more predominant foodborne serovars throughout the world (Jay 2000) and is consistently documented as being responsible for foodborne disease outbreaks. As a primarily intestinal organism, cells are excreted in the feces of infected animals and can potentially spread to other locations. Although infection can occur experimentally

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when a host ingests sufficient cells (Blaser and Newman 1982), exact minimal dosage numbers that occur in documented outbreaks have proven elusive. After the bacterial cells have penetrated into the host's cells, symptoms of the disease begin to manifest within the cell. Clinical symptoms consist of nausea, vomiting, abdominal pain, headache, chills and diarrhea that may persist for approximately 2–3 days (Jay 2000). However, symptoms may last for weeks depending on the attack rate of the organism and the specific serovar (Kothary and Babu 2001). Symptoms of *Salmonella* food poisoning are generally mild, but can be quite severe to young or elderly individuals and also individuals who are immunocompromised. The U.S. economy loses approximately $4bn a year resulting from *Salmonella* infections through the added costs of clinical treatment and medications (Todd 1989).

FOODBORNE *SALMONELLA* PATHOGENESIS AND VIRULENCE EXPRESSION

During a *Salmonella* infection, cells invade the apical surface of a host's enterocytes within the intestine (Jones *et al.* 1994). Once the bacteria come in contact with the enterocytes, a series of changes occur in the host cells. The entry of *Salmonella* is facilitated through actin rearrangement and ruffling of the cell membrane resulting in cellular projections that envelope the bacteria (Finlay and Falkow 1997). This internalization mechanism is complicated and requires signaling between the host cell and the bacterial cell. Membrane ruffling is actually the ability of *Salmonella* cells to denature the proteins within the microvilli of the small intestine (Finlay and Falkow 1990). When the *Salmonella* becomes internalized within the host cell, it remains in a membrane-bound compartment for the duration of the intracellular life cycle. After remaining in the host cell for approximately 4 h, *Salmonella* has the ability to replicate in nonphagocytic cells (Galán and Sansonetti 1996). Once successfully inside the host cell, replication begins and *Salmonella*-induced lysis of the epithelial cell will occur within 10–16 h. Cells will then begin to migrate to the mesenteric lymph nodes as well as organs such as the spleen and liver (Richter-Dahlfors *et al.* 1997).

Bacterial virulence requires a myriad of factors to be present for many pathogenic species. The concept of bacterial virulence is the means by which the organism ultimately causes disease within the host (Mekalanos 1992). *Salmonella's* invasion phenotype appears to be highly regulated and assembly of specific invasion factors requires coordinate regulation of genes that encode these components. Virulence factors usually do not contribute to the bacteria's cell structure or function. The expression of virulence factors is likely to occur shortly after the cell has been introduced into a host to maximize successful infections (Mahan *et al.* 1996). The surrounding environment changes drastically once *Salmonella* cells penetrate host tissue. Changes also occur in the bacterial cell once they come in contact with cells in the small intestine. Because of these changes, it appears that salmonellae regulate their invasiveness by releasing specific virulence factors only at the time when the bacteria interact with host cells (Bajaj *et al.* 1996). Specific genes expressed when *Salmonella* contact and enter epithelial cells in the small intestine have been identified (Altier and Suyemoto 1999). It is now understood that more than one virulence factor is involved in the pathogenesis of the host to parasite interaction. It is thought that the expression of other virulence factors may be coordinately controlled by one common regulatory system (Mekalanos 1992).

Salmonella invasiveness is also regulated by specific environmental and bacteria growth conditions such as oxygen availability, osmolarity, and pH (Bajaj *et al.* 1996). Other factors regulating virulence expression include iron and calcium availability or stress on the bacterial cell (ex. starvation or heat shock) (Mekalanos 1992). Environmental stresses such as starvation may also control the expression of virulence genes. The expression of genes required for survival of *Salmonella* within macrophages is controlled by the two-component regulatory system PhoP-PhoQ that includes the regulator PhoP, the kinase–phosphatase PhoQ, and several PhoP-regulated genes (Fields *et al.* 1989; Groisman and Heffron 1995). Based on a series of experiments, it appears that this system responds to carbon and nitrogen starvation conditions and indicates that PhoPQ may respond to environmental conditions (Mekalanos 1992). The PhoPQ system is also regulated by low extracellular cation levels which *Salmonella* cells may encounter within macrophages (Bajaj *et al.* 1996).

GENETICS OF FOODBORNE *SALMONELLA* SPP. VIRULENCE

A majority of *Salmonella's* invasion genes which interact with the epithelial cell are encoded on a 40-kb region known as *Salmonella* pathogenicity island 1 (SP1) close to minute 63 of the *S.* Typhimurium chromosome (Mills *et al.* 1995). *Salmonella* pathogenicity islands are defined as large clusters of genes in a chromosome that encode factors responsible for interactions with the host and are required for virulence (Marcus *et al.* 2000). There are five pathogenicity islands and SP1 has been shown to be primarily responsible for *Salmonella* penetration into host epithelial cells within the intestine (Marcus *et al.* 2000). The invasive role of SP1 has been confirmed through a series of experimental studies. In short, it was demonstrated that *S.* Typhimurium SP1 mutants were attenuated for virulence when inoculated orally, but not systemically when injected into mice (Galán and Curtiss 1989).

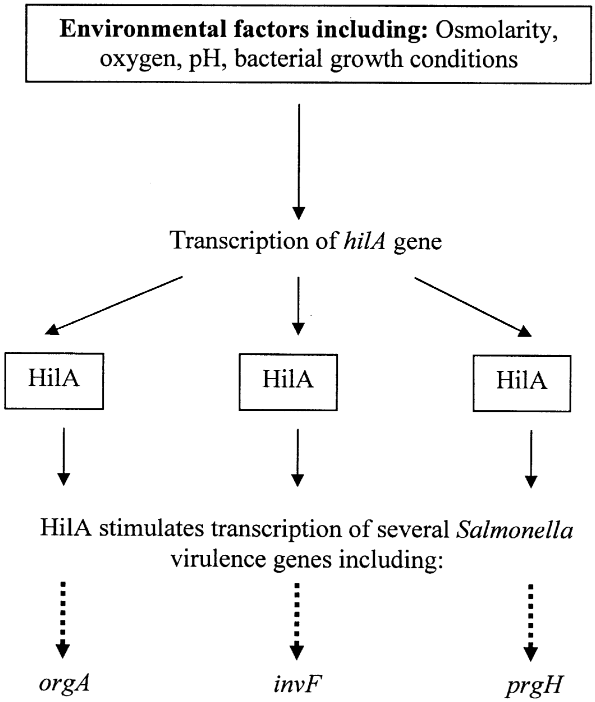

FIG. 1. EVENTS LEADING TO THE COORDINATE REGULATION OF VIRULENCE GENE EXPRESSION. THE CONTROL OF *hilA* IS REGULATED BY ENVIRONMENTAL FACTORS AND THE HilA PROTEIN STIMULATES THE TRANSCRIPTION OF OTHER VIRULENCE GENES
Figure depiction simplified from Bajaj *et al.* (1996).

It has been reported that *Salmonella's* genome may contain as many as 200 virulence genes (Bowe *et al.* 1998). Of these genes, several have been extensively studied and their role in pathogenesis is now somewhat understood. A gene located in SP1 named *hilA* (Hyper Invasive Locus) encodes a transcriptional activator and is essential for *Salmonella* invasion (Bajaj *et al.* 1995). The transcriptional activator encoded by *hilA* (HilA) is a protein that is required for the initiation of RNA synthesis at a specific promoter. The HilA protein is approximately 60–63 kDa and is similar to the DNA binding and transcription activation system of the OmpR/ToxR family of regulators (Bajaj *et al.* 1995). HilA is similar to other groups of transcriptional activators in that it regulates genes in response to physiological conditions and is involved in prokaryotic signal transduction (Stock *et al.* 1989). The expression of *hilA* is also stimulated by the same environmental conditions (oxygen, osmolarity, pH) that regulate invasiveness in *Salmonella* (Fig. 1; Bajaj *et al.* 1996). Thus, HilA appears to be involved in some fashion with the stimulation of other genes required by *Salmonella* to become invasive. More specifically, it has been determined that HilA is required for the maximum expression of at least three essential invasion genes: *invF, sspC* and *orgA* (Bajaj *et al.* 1995). The response that HilA elicits on the expression of these virulence genes may be direct or indirect.

The regulation of HilA and the mechanism of how HilA induces these virulence genes has been investigated and is quite complex (Darwin and Miller 1999; Lucas and Lee 2000; Schechter and Lee 2000). However, in this complex scheme, HilA still appears to play a central role. Research by Bajaj *et al.* (1996) has shown that HilA acts directly at the promoters of specific invasion genes to activate expression in *Salmonella*. Because the *hilA* gene clearly plays an important role in *Salmonella* pathogenicity, the activity of this gene can be used as an overall indicator of virulence gene expression. This is based in part on the demonstration that mutations in the *hilA* gene result in a dramatic loss of the cell's ability to become invasive, thus demonstrating the importance of *hilA* in the invasion phenotype (Penheiter *et al.* 1997). However, the mechanism of how specific environmental signals regulate the expression of *hilA* and other invasion genes is still relatively unknown. Several possibilities of how bacteria sense these physiological cues and respond to them to generate the invasive phenotype have been investigated (Bajaj *et al.* 1995). In addition to being required for specific invasion gene expression, *hilA* also modulates the expression of the type III secretion system (Fahlen *et al.* 2000).

Salmonella uses a type III protein secretion system for delivery of virulence gene products (Collazo and Galán 1997). This system is designed as a complex assembly of proteins that span the inner and outer membranes. The type III secretion system is tightly regulated and only allows proteins to be secreted when the bacteria encounter specific environmental cues (Marcus *et al.* 2000). Outer membrane proteins identified in *Salmonella* include the InvG, PrgH and PrgK proteins (Kaniga *et al.* 1994). It is believed that InvG plays a key role in bacterial uptake and in protein secretion (Kaniga *et al.* 1994). Inner membrane proteins of the type III secretion system include InvA, SpaP, SpaQ, SpaR and SpaS proteins (Galán *et al.* 1992; Ginocchio *et al.* 1994; Collazo and Galán 1996). The *spa* genes play a role in *Salmonella* entry into cells as mutations in this gene prevent cell invasion and other protein secretion (Collazo and Galán 1997).

IN VITRO ASSAYS FOR ASSESSING *SALMONELLA* VIRULENCE GENE RESPONSE

The environment that enteric pathogens encounter within the gastrointestinal tract is important when understanding the expression of virulence genes.

Some bacterial genes are actually triggered when present in these environments and as a result can cause a more effective colonization within their host. The expression of these certain genes may actually increase the rate of infectivity of the bacteria and prolong the duration of the illness. Determining what causative agents stimulate or repress bacterial virulence gene expression can potentially decrease the incidence of this hyper-infective state of bacterial cells, thus helping to reduce foodborne illness.

By measuring the activity of certain enzymes or the product of detectable biological reactions, the expression of bacterial genes whose products are difficult to detect can be quantitated as an enzyme assay. This in turn can be used to quantitatively monitor gene expression and predictions can be made regarding the potential for which environmental conditions may lead to heightened levels of pathogenesis. Reactions that yield colored compounds, fluorescent compounds, or even bioluminescence are typically used to monitor cell physiology and genetic expression. The use of specific reporter enzymes requires that the enzyme meet two basic requirements. A reporter enzyme must possess an activity distinctly different from the bacterial enzymes used in the system, and the enzyme assay must be extremely sensitive to generate conclusive experimental results (Pfeifer and Finlay 1995).

A key to understanding the genetic principles of bacterial virulence is to monitor gene expression throughout the invasion process. However, products produced by virulence genes are difficult to quantitatively detect in conventional assays. The application of gene fusions and specific fusion strains of bacteria have been used to detect and/or monitor many biological functions. A gene fusion is defined as the attachment of the amino terminal portion of a specific protein to the carboxy terminal portion of another while maintaining the functional activity of one or both proteins (Silhavy and Beckwith 1985). Genetic fusions of previously described reporter enzymes and fluorescent compounds have been used to assay specific genes necessary for virulence determinants.

β-GALACTOSIDASE AS A REPORTER ENZYME

The β-galactosidase enzyme is commonly used in many molecular and bacteriological experiments as a reporter enzyme (Pfeifer and Finlay 1995). The function of this enzyme is to hydrolyze β-D-galactosides. More specifically, it converts lactose into galactose and glucose in biological systems. The β-galactosidase protein is a tetrameric protein comprised of identical subunits. Gene fusion analysis has shown that up to 26 amino acids can be removed from the amino terminus and replaced with other sequences, which overall has very little effect on the enzyme's activity (Brickman *et al.* 1979). The genes that code for the production of β-galactosidase are known as *lacZ* and *lacY*.

Incorporation of *lacZ* gene fusions has been very helpful in providing an insight into the analysis of bacterial genomes (Derbyshire 1995). A major advantage of using the *lac* operon is that it is one of the most extensively studied genetic systems. Many genetic and biochemical aspects of this system are known and can be used in laboratory settings (Silhavy and Beckwith 1985). Another benefit of using the β-galactosidase enzyme in molecular experiments is that its activity can be measured with chromogenic substrates, colorless compounds which when hydrolyzed produce colored products. An example of such a substrate is *o*-nitrophenyl-β-D-galactosidase (ONPG). This is a colorless compound, however, in the presence of β-galactosidase it is converted to galactose and *o*-nitrophenol. The *o*-nitrophenol is yellow and can be measured in a spectrophotometer, thus estimating the overall activity of the enzyme (Miller 1972). The optical density values are incorporated into a standardized equation that yields a numerical value representing the degree of *hilA* expression. Disadvantages of using this assay include time constraints and the inability to analyze several samples at one time. Adaptation of tube-based assays to microtiter plates and standardization of media controls have provided a means to reduce the assay time and scale down the quantities of reagents required to implement a more routine application of the assay to large numbers of samples (Nutt *et al.* 2002, 2003).

LUCIFERASE

The luciferase enzyme (*lux* operon) obtained from a species of soil bacteria named *Photorhabdus luminescens* and can be inserted with relative stability into enterobacteriaceae to serve as a receptor gene (Francis and Gallagher 1993; Lee and Camilli 2000). A major advantage of using luciferase to monitor gene expression is that this enzyme's substrate, luciferase aldehyde, is capable of crossing cell membranes (Park *et al.* 1992). This is helpful because the bacterial cell or host being assayed is not required to be lysed. Measuring luciferase activity is extremely accurate because a large majority of bacteria have no endogenous luciferase activity. Therefore, any luciferase activity detected is the result of the expression of the bacterial gene tagged with the enzyme (Pfeifer and Finlay 1995). Detecting the amount of bioluminescence produced is usually performed using an intensified charged-couple device (ICCD) camera detector which amplifies the bioluminescent signal (Lee and Camilli 2000).

This device has been used to successfully monitor the adherence of *Escherichia coli* O157:H7 cells to cattle carcass tissue (Siragusa *et al.* 1999).

Bioluminescence techniques have been used in food microbiology for a variety of applications (Baker *et al.* 1992; Pietrzak and Denes 1996). Bioluminescence methods have been specifically used to detect and model *S.* Typhimurium thermal inactivation (Duffy *et al.* 1995) and transcriptional *hilA* fusion *S.* Enteritidis strains have been constructed for monitoring virulence response to medium chain fatty acids (Van Immerseel *et al.* 2004).

GREEN FLUORESCENT PROTEIN

Another method of monitoring the physiologic or genetic state of a bacterial cell is through the use of the green fluorescent protein gene (*gfp*). The *gfp* gene was first isolated from the jellyfish *Aequorea victoria* and was later cloned (Prasher *et al.* 1992). Green fluorescent protein (GFP) has been used to study the genetic expression and chromosome segregation in prokaryotes and eukaryotes (Lee and Camilli 2000). The natural function of this protein is to convert the blue light signal of the calcium sensitive photoprotein aequorin into the detectable green light emission (Cody *et al.* 1993). A major advantage of using GFP is that it requires no substrate or other cofactors to induce fluorescence (Chalfie *et al.* 1994). The cells that are tagged with the *gfp* gene are also quite easy to visualize and detect by the use of epifluorescence microscopy. Visualization of the tagged cells can be achieved with just a single copy of *gfp* integrated into the bacteria chromosome (Suarez *et al.* 1997). Historically this approach has been quite useful when trying to determine the presence of organisms that may not be readily detectable by conventional means. Transcriptional *gfp* fusion *S.* Enteritidis strains have been constructed for several virulence genes to examine gene expression during infection of mammalian tissue culture cells (Hautefort *et al.* 2003).

CONCLUSIONS

A variety of different factors are involved in pathogenesis when foodborne *Salmonella* spp. are ingested into a host. However, the mode of infection and the necessity of certain genes to facilitate bacterial invasion are important factors in combating disease and ensuring public safety. Therefore, understanding the mechanisms that specific bacteria undergo to become pathogenic may potentially reduce the numbers of infectious disease. Depending on the environment and genetic state of *Salmonella*, some cells may be more prone to infect and ultimately cause disease.

Determining the specific factors that stimulate overall *hilA* expression and ultimately other virulence genes of *Salmonella* is important and can potentially be used to decrease the incidence of foodborne infection. Implementation of several detection technologies provide the means to develop and apply rapid assays for quantifying virulence foodborne *Salmonella* spp. gene expression using specifically constructed transcriptional fusion strains as indicator organisms. Developing the capacity to monitor expression of foodborne *Salmonella* spp. virulence genes during food production and processing will evolve into an important aspect for assessment of risk.

ACKNOWLEDGMENTS

This review and J.D.N. were supported by the Texas Higher Education Coordinating Board's Advanced Technology Program (#000517-0361-1999), USDA-NRI grant number 2001-02675, and Hatch grant H8311 administered by the Texas Agricultural Experiment Station.

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